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Skin Oxygenation After Topical Application of Liposome-Entrapped Benzyl Nicotinate as Measured by EPR Oximetry in vivo: Influence of Composition and Size

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ABSTRACT

New and improved drug delivery systems are the important subject of much scientific research. The development of formulations that increase skin oxygenation and of methods for measuring oxygen levels in skin are important for dealing with healing processes affected by the level of oxygen. We have used EPR oximetry in vivo to compare the influence of liposomal formulations of different size and composition with that of hydrogel with respect to the action of the entrapped benzyl nicotinate (BN). Following the topical application of BN onto the skin of mice, pO_2 increase was measured by low-frequency EPR as a function of time. The effect of BN was evaluated by 3 different parameters: lag-time, time needed for maximum pO_2 increase, and overall effectiveness expressed by the area under the response-time curve. An increase in skin oxygenation was observed after BN application. The results show that the effect of BN incorporated in liposomes is achieved more rapidly than the effect from hydrophilic gel. The composition of the liposomes significantly affects the time at which BN starts to act and, to a lesser extent, the maximum increase of pO_2 in skin and the effectiveness of BN action. However, the size of the liposomes influences both the effectiveness of BN action and the time at which BN starts to act. After repeated application of liposomes, the pO_2 baseline increased and the response of the skin tissue was faster. Our results demonstrate that EPR oximetry is a useful method for evaluating oxygen changes after drug application and for following the time course of their action.

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KEYWORDS: skin, liposome, benzyl nicotinate, oxygenation, EPR in vivo

INTRODUCTION

Improved skin oxygenation has a positive effect on the treatment of ischaemic diseases. It stimulates the healing process of damaged skin and increases the effectiveness of radiotherapy in skin cancer treatment.[1,2] The partial pressure of extracellular oxygen (pO_2) influences a number of cellular functions, including growth and metabolism.[3] The pO_2 in healthy skin varies from 10 to 40 mmHg, compared with pathological processes such as tumors and hypoxic wounds where oxygen levels fall below 5 mmHg (the true ischemia).[2,4] The radiosensitivity of cells is greatly affected by the local concentration of oxygen and increases dramatically in the range from 0 to 10 mmHg, where saturation has been observed.[5] Topical application of vasodilators that increase skin oxygenation could therefore enhance the effect of radiation on tumors.

Nicotinate esters are suitable candidates for studying such effects. They act as prodrugs, which cross the skin rapidly and, on enzymatic hydrolysis, release nicotinic acid. This agent provokes increased cutaneous blood flow, at least partly by forming vasodilating prostaglandins.[6] As a consequence of the dilatation of small arterioles, the skin color changes and the level of oxygen in skin increases. The time when maximal effect is achieved and the duration of vasodilation depend on the concentration of the drug and its chemical structure (nicotinic acid and different esters: methyl, ethyl, hexyl, benzyl, tetrahydrofurfuryl).[7-9] The rate of rubefacient action, as well as its effectiveness, depends not only on the rubefacient used but also on the carrier in which the rubefacient is applied.[10-12]

Liposomes are drug carriers for dermal therapy, which can considerably improve the effectiveness of drugs and at the same time diminish their side effects.[11-13] Although there is general agreement that liposomes as drug carriers enhance the penetration of drugs through the skin, little is known about the actual mechanisms of interaction among liposomes, drug molecules, and the stratum corneum. The mechanism by which liposomes affect the penetration of drugs into the skin is not completely understood.[14,15] The structural requirements of lipids for optimal lipid mixing with human stratum corneum have been addressed.[16] Several studies show that liposome composition and, to a lesser extent, liposome size influence the rate of transport and effectiveness of drug action in skin. The results were based primarily on in vitro measurements[17-19] or on qualitative in vivo data.[20,21] However, there is no method that would enable the response of an organism to a drug to be measured directly and quantitatively and, hence, the effectiveness of different types of liposomes or other formulations to be assessed in vivo.

The penetration through the skin of nicotinates incorporated in different formulations has been studied by various physical methods.[22,23] However, they do not yield quantitative results because they are not able to correlate directly the blood flow characteristics with oxygen levels in skin. Electron paramagnetic resonance (EPR) enables in vivo quantitative measurement of the response of an organism to the action of topically applied rubifacient.[12,24] The method is based on the fact that molecular oxygen is paramagnetic and causes fast relaxation of other paramagnetic species by Heisenberg spin exchange interaction. As a consequence, the spectral line-width of the paramagnetic probe is broadened, to an extent that depends on the oxygen concentration.[25,26] The development of low frequency EPR spectrometers (less than 1.2 GHz) with surface coils makes it possible to measure pO_2 in vivo.[27] After initial insertion of the paramagnetic probe into the tissue, measurements are non-invasive. The method allows pO_2 to be monitored repeatedly at the same point in a tissue, over the entire period of treatment.

To investigate the effects of liposome composition and size on the effectiveness of benzyl nicotinate (BN) penetration into the skin and subsequent oxygenation, we measured the processes quantitatively in vivo by EPR oximetry. BN was incorporated into liposomes containing hydrogenated (HSL) and non-hydrogenated (NSL) soybean lecithin and cholesterol, and the overall effectiveness of the entrapped drug was determined. Free BN in hydrogel was used as a control formulation. The time course and extent of the nicotinate reaction were quantified in terms of cutaneous oxygen concentration. In this way, it has been possible to assess whether the results previously obtained in vitro can be applied to in vivo conditions and, at the same time, to determine the types of liposome that would improve the action of BN on skin.

MATERIALS AND METHODS

Materials

BN was obtained from Lek (Ljubljana, Slovenia), hydroxyethylcellulose (Natrosol® 250 HHX) from Aqualon (Wilmington, Germany), glycerol from Pharmachem (Ljubljana, Slovenia), HSL (Emulmetic® 320) from Lucas Mayer (Hamburg, Germany), NSL (Phospholipon 80®) from Natterman Phospholipid GmbH (Cologne, Germany), and cholesterol (Ch) from Merck (Darmstadt, Germany). The paramagnetic probe lithium phthalocyanin (LiPc) was a kind gift from the EPR Center for Viable Tissues, Dartmouth Medical School (Hanover, NH).

Hydrogel with BN

Hydroxyethylcellulose, 2.0 g, was dispersed in two thirds of the total mass (77.17 g) of distilled water at 70°C, then cooled to room temperature with continuous stirring to give a homogeneous mixture. BN, 0.83 g, dispersed in 20.0 g glycerol was added, together with the hydroxyethylcellulose dispersion, to the remaining water and mixed to give homogeneously dispersed BN in hydrogel.

Liposomes with BN

Liposomes were prepared by the thin film method from Ch and either HSL or NSL in a weight ratio of 3:7. The lipophilic phase containing phospholipid, together with Ch and BN, was dissolved in dichloromethane for NSL or in chloroform:methanol (1:1) for HSL. The solvent was removed in a rotary evaporator to give a thin film on the wall. Remaining solvent was removed completely under vacuum (10 to 15 minutes at 40ºC and pressure 100 Pa). The dry film was hydrated with distilled water at approximately 80ºC for HSL (ie, above its phase transition temperature) and at room temperature (22ºC) for NSL. The flask was shaken until the film was completely removed from the walls. The liposome dispersion was stabilized by stirring for 2 hours on a magnetic stirrer (300 rpm) at room temperature. A 1-

mL sample of liposome dispersion contained 25 mg of lipids and 12.5 mg of BN. Liposomes could not be formed at higher concentrations of BN.

A portion of the multi-lamellar liposomes (MLV) was extruded through a Liposofast extruder (Avestin, Ottawa, Canada) using polycarbonate membranes with defined pores from 800 to 100 nm (Nucleopore Corporation, Pleasation, CA). The liposomes were extruded at temperatures above the phase transition temperature.

Characterization of liposomes

Liposome size and polydispersity index (PI) were determined by photon correlation spectroscopy (PCS; Zetasizer 3000, Malvern, Malvern, UK) at a fixed angle of 90º. Samples were diluted with dust-free water to give the recommended scattering intensity of 100 000 counts s^{-1}. The diameter was calculated from the autocorrelation function of the intensity of light scattered from particles, assuming a spherical form of particles. For mean size calculation, the cumulant algorithm, which takes into account only 1 population of particles, was used. The PI is a measure of dispersion homogeneity, which ranges from 0 (homogeneous dispersion) to 1 (high heterogeneity).

Liposomes with BN in hydrogel

A 2.0-g amount of hydroxyethylcellulose was dispersed in water (12.0 g) together with one third of the liposome dispersion (66.0 g) at room temperature. After 1 hour, 20.0 g of glycerol and the remaining liposome dispersion were added and mixed to yield a homogeneous hydrogel. The amount of BN free in hydrogel or entrapped in liposomes was 0.83% (wt/wt).

Experimental animals

Guidelines and legislative regulations on the use of animals for scientific purposes were followed. Female Balb C mice were housed for at least 1 week in the animal facility before the start of experiments. The animals received standard laboratory chow and water ad libitum and weighed between 20 and 25 g. The hair of the dorsal area was cut off, and the remainder removed with a depilatory cream (Vitaskin®; Krka, Novo mesto, Slovenia). Measurements were made 4 days after depilation, when the stratum corneum had completely recovered.[28]

One day before the experiment the mice were placed, 1 per cage, in cages that had been modified to avoid contact with the application zone on the back. The mice were anesthetized by intraperitoneal injection of a mixture containing xylazine (Rompun® 2%; Bayer, Leverkusen, Germany) 16 mg/kg and ketamine (Ketanest® 50; Parke-Davis, Berlin, Germany) 100 mg/kg. During measurements, an additional dose of anesthetic was added when needed.

In vivo EPR experiments

Amorphous particles of LiPc were inserted into the mouse skin through an injection needle (23 G). Because the decrease of body temperature of the mice following anesthesia could significantly influence the line-width of the EPR spectra,[29] the body temperature was maintained at 36.5 ± 0.5°C by a flow of hot air, and was measured rectally with a thermocouple inserted into a glass capillary. The mouse was anesthetized and the EPR spectral line-width measured for 15 minutes to obtain the baseline pO_2 in the skin.

Hydrogel with BN was then applied to the skin using a plastic ring, 1-mm thick with a hole of 18-mm diameter. The ring was placed on the mouse skin and the hole filled with the formulation. The ring was then removed but the formulation remained on the site of application during the measurements. EPR spectra were recorded continuously for at least 90 minutes and the line-width of the spectra measured to give the pO_2 at the site of the paramagnetic probe. Formulations containing no BN were used as a controls.

EPR measurements

The measurements were performed on a Varian E-9 EPR spectrometer (Varian, Palo Alto, CA) with a custom-built low-frequency microwave bridge operating at 1.1 GHz with a surface coil detector (11-mm diameter), both designed by Dr T. Walczak (Dartmouth Medical School, Hanover, NH). Of the different paramagnetic probes sensitive to oxygen, LiPc was found to be the most suitable for our experiments. The signal-to-noise ratio of LiPc is good, even at higher physiological pO_2, and consequently the measurements are precise over a greater range of oxygen concentration. Twenty-one days after insertion into tissue LiPc loses its responsiveness to oxygen. Measurements were made, therefore, for only 14 days after insertion into the skin. Optimal spectrometer settings for LiPc were determined at modulation frequency 100 kHz, modulation amplitude not exceeding one third of the peak-to-peak line-width (0.002-0.005 mT), scan range 0.4 mT, and scan time 60 seconds.

Changes in pO_2 were determined by measuring the peak-to-peak line-widths of the EPR spectra (ΔB, **Figure 1**). The relation between pO_2 and line-width was calculated from equation 1 obtained from the published calibration curve[29]:

$$pO_2 = 2.56x10^3 \Delta B - 14.97 \quad (1)$$

pO_2 is in mmHg and ΔB in mT.

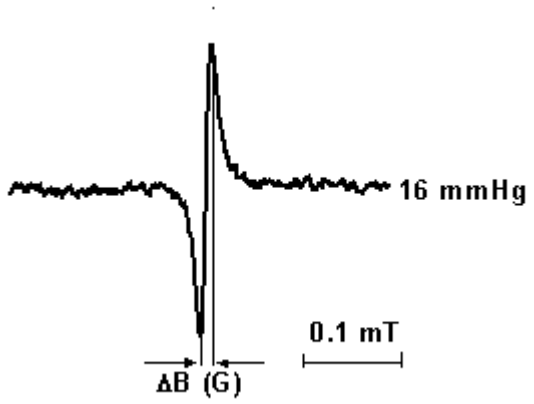

Figure 1. Typical EPR spectrum for LiPc paramagnetic probe at 16 mmHg partial pressure of oxygen with the peak to peak line-width indicated (ΔB).

Statistical evaluation

Data are reported as arithmetic means ± standard error of mean (SEM), with n ≥ 5. Comparisons were performed by Student *t*-test. Significance was tested at the .05 level of probability.

RESULTS AND DISCUSSION

Effect of different formulations on pO_2 in skin

Experiments on all applied formulations without BN were carried out to investigate their influence on skin oxygenation. Hydrogel and liposomes, in the absence of BN, do not cause a change in oxygen concentration in the skin. The lag-time (t_{lag}), the time from application of the formulation to the first increase of pO_2; t_{max}, the time for achieving maximal increase in pO_2; AUC, the area under the response-time curve; and ΔpO_2, the maximal increase in pO_2 after application of BN, were determined from the individual pO_2 curves as shown in **Figure 2**. The baseline of pO_2 varies from animal to animal from 6 to 20 mmHg, due to the physiological state of the mouse, the vessel's dilatory ability, and different locations of LiPc in the skin. The difference, ΔpO_2 , between the pO_2 baseline and the measured pO_2 after application was therefore derived for each mouse experiment. The influence on the oxygenation of the skin of free BN in hydrogel and of BN delivered at the same concentration in HSL or NSL liposomes is shown in **Figure 3**.

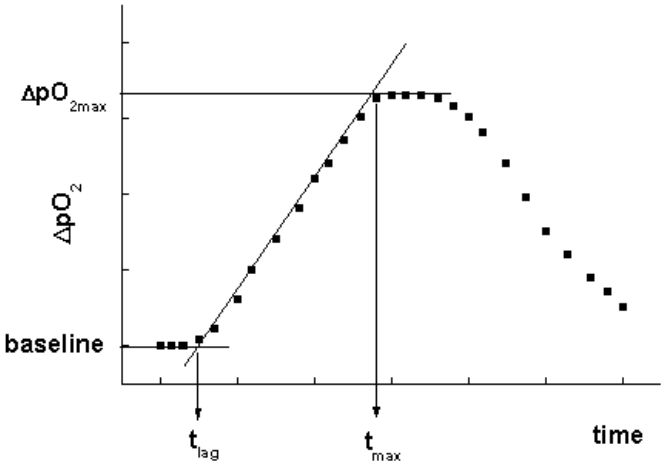

Figure 2. Schematic response-time curve of skin tissue after BN application with indicated parameters.

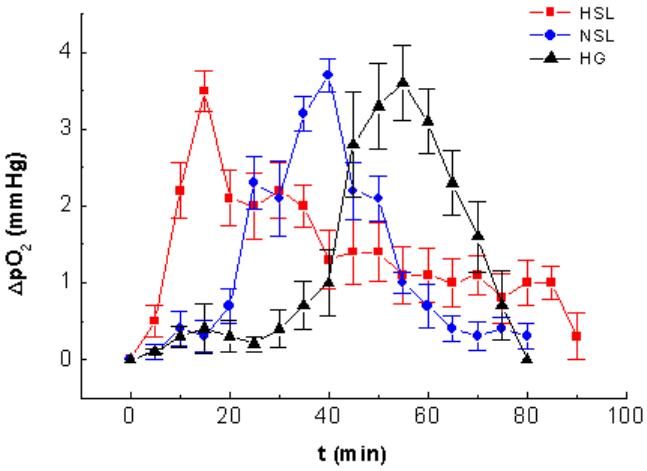

Figure 3. The time course of oxygen level (pO_2) in mouse skin after application of 0.83% wt/wt benzyl nicotinate in different vehicles. (—■—) HSL and (—●—) NSL liposomes in hydrophilic gel (both non-extruded) and (—▲—) hydrophilic gel. Each point represents the mean ± SEM of 5 measurements on at least 5 mice. The initial delay (lag-time) is not shown and is presented in Table 1.

Table 1. Influence of Different Vehicles on the Oxygenation of Mouse Skin*

BN *in*		mean diameter (nm)	ΔpO_{2max} (mmHg)	t_{max} (min)	t_{lag} (min)	AUC_{0-80} (mmHg × min)
Hydrogel		—	3.6 ± 0.5	55 ± 2	39 ± 2	90 ± 24
NSL	**NE**†	349 ± 39	3.7 ± 0.2	40 ± 1	17 ± 1	117 ± 6
	E‡	250 ± 21	3.9 ± 0.6	78 ± 1	40 ± 3	110 ± 9
HSL	**NE**†	773 ± 268	3.5 ± 0.3	17 ± 1	9 ± 1	121 ± 17
	E‡	282 ± 8	3.3 ± 0.4	17 ± 4	10 ± 4	58 ± 11

*Benzyl nicotinate (BN) was applied topically, free in hydrogel (HG), or entrapped in hydrogenated (HSL) and non-hydrogenated (NSL) soybean lecithin liposomes.

† Non-extruded.

‡ Extruded (see text).

ΔpO_{2max} is the maximal relative increase of pO_2 after application of the formulation, t_{lag} is the time between the application of the formulation and the time when pO_2 starts to increase, t_{max} is the time when the maximal increase in pO_2 is achieved, and AUC is the area under the curve ($= 1/2\ \Sigma\ (t_{i+1} - t_i)\ (pO_{2i} + pO_{2i+1})$, where t_i is the time of measurement and pO_{2i} is the corresponding partial pressure for i = 0 – 80). Each value is the mean ± SEM of measurements on at least 5 mice. The differences in the lag-time and t_{max} between different carriers (hydrogel, NSL and HSL liposomes) are statistically significant, while all other parameters measured (ΔpO_2 and AUC) are not significantly different on the probability level > .05.

Different carriers significantly affect the time when maximal pO_2 is achieved and the lag time (**Table 1**). Both are significantly shorter for the 2 types of liposome than for BN free in hydrophilic gel. Liposomes enable penetration of the encapsulated drug closer to the vessels in the dermis, where the main site of action of the drug is. The diffusion path of BN is therefore shortened and the effect appears sooner. For the same reason, the time needed to reach the maximum effect is longer for BN freely dispersed in hydrogel.

In contrast to the time response of the organism to drug action, which depends strongly on the carrier, no significant difference in the maximal increase in pO_2 or in the effectiveness of BN in different carriers was observed. This is not surprising since BN is a well-known vasodilator that, as a lipophilic substance, can penetrate through the stratum corneum (SC) barrier. Therefore it could be expected that the main influence of the different carriers is to accelerate its action, or in some cases influence either the rate of release from liposomes or drug diffusion (barrier structure), as seen for multilamellar HSL liposomes. This study suggests that the liposomes have advantages for increasing the penetration rate for lipophilic, and not only hydrophilic, substances into the skin.

Effect of liposome composition

At the temperature of skin, 32°C, the membranes of HSL liposomes (which contain 30% Ch) are in the phase where solid-ordered (gel) phase and liquid-ordered phase coexist, while NSL liposomes are in liquid-disordered phase.[30] The former have more rigid bilayers than those in liquid-state. These liposomes were used to study the effect of the thermodynamic state of the bilayer on the penetration of the model drug into the skin. The action of BN applied in the more rigid HSL liposomes occurred after 9 minutes, with t_{max} at 17 minutes, while BN in NSL liposomes was first detectable after 17 minutes, with t_{max} at 40 minutes (**Table 1**). It was typical for multi-lamellar HSL liposomes that after the maximum was reached, the pO_2 decreased only to a certain level, which was maintained for a prolonged period, indicating sustained release of the drug. These results confirm our previous findings of in vitro and in vivo EPR experiments where we investigated the influence of liposome composition on the bilayer fluidity and the transport of encapsulated substance into the skin.[17,28,31] The results indicate that, in the first 30 minutes, HSL liposomes enable the transport of entrapped hydrophilic probe deeper into the skin, but NSL liposomes only into the stratum corneum. This explains the difference in the time at which maximal pO_2 was achieved.

The difference in penetration depth of locally applied more rigid and liquid-state liposomes was also observed in the in vitro confocal laser scanning microscope study on rat skin.[21] Liposomal phospholipids applied onto the skin form a steep concentration gradient from the skin surface to the deeper layers. In order to explain the deeper penetration of the drug, it is reasonable to expect that the carrier phospholipids, by adsorption and fusion with the stratum corneum, disrupt its barrier function. Successful percutaneous delivery of entrapped molecules relies strongly on an adequate reduction of the barrier properties of the stratum corneum, which is considered to constitute the main barrier of the skin. Our findings are additionally supported by in vitro studies using freeze-substitution electron microscopy, which showed that the upper lipid bilayers of the cell were occasionally disrupted after applying gel-state liposomes.[15]

Effect of liposome size on the transport of BN

The mean diameters of HSL and NSL liposomes prepared by the same method, both with and without extrusion, are presented in **Table 1**. After extrusion, the size was reduced until it reached similar values for NSL and HSL liposomes. The changes in pO_2 after applying extruded HSL and NSL liposomes are presented in **Table 1** and **Figure 4**. For both types of liposome, the size of the liposomes affected the response of the tissue to the drug, but in different ways. For HSL, the time response remained almost the same, but no sustained release was observed. The effectiveness of drug action was thus decreased by using a population of smaller, more homogeneous liposomes. It appears that mainly uni-lamellar liposomes had a more narrow range of stabilities, so that all the liposomes break down at the same time in the skin, releasing the whole amount of the drug over a shorter time period. Therefore the concentration of BN was higher than with non-extruded HSL liposomes, but the pO_{2max} cannot further increase, since the vessel's maximal dilatation is already achieved.

Extruded NSL liposomes behave differently. The time at which maximal effect was achieved was significantly later than for the non-extruded liposomes, but ΔpO_{2max} and the effectiveness of BN action did not change significantly. It was shown[17] that, after extrusion to about 200-nm diameter, NSL liposomes become very unstable. Therefore we assume that they break down immediately on contact with skin and they therefore act similarly to BN applied free in hydrogel.

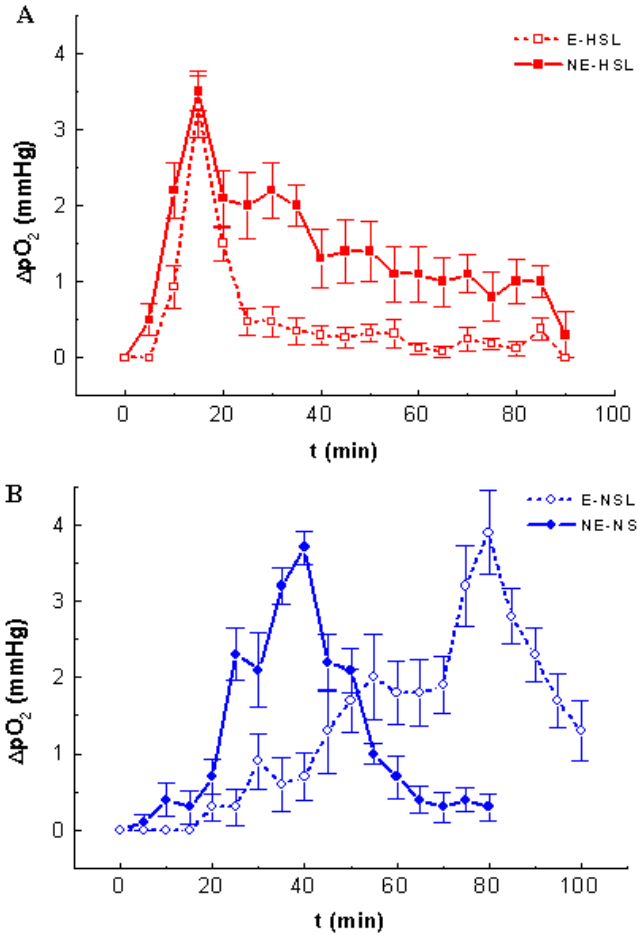

Figure 4. The time course of oxygen level (ΔpO_2) in mouse skin after application of benzyl nicotinate in HSL (A) and NSL (B) liposomes of different sizes: (—■—) non-extruded HSL liposomes, (---□---) extruded HSL liposomes, (—●—) non-extruded NSL liposomes, (---○---) extruded NSL liposomes. Each point represents the mean ± SEM of 5 measurements on each of at least 5 mice.

Effect of repeated application

Topical formulations containing nicotinate esters are usually applied to the skin repeatedly over a longer period. The effect of repeated application of non-extruded HSL liposomes once a day was evaluated over a period of 5 days (**Table 2** and **Figure 5**). After each successive application of BN, the pO_2 baseline increased by 1 mmHg on the second day and by 2.5

Table 2. The Effect of Daily Repeated Topical Application of Benzyl Nicotinate (BN) Entrapped in Non-Extruded Hydrogenated Soybean Lecithin (HSL) Liposomes on Oxygenation of the Mouse Skin Over a Period of 5 Days*

Time (Days)	Daily Baseline (mmHg)	$\Delta pO_{2\,max}$ (mmHg)	t_{lag} (min)	t_{max} (min)
1	13.9 ± 0.1	3.1 ± 0.1	10	19
2	14.9 ± 0.4	3.2 ± 0.2	7	18
3	16.4 ± 0.6	5.1 ± 0.2	6	10
4	18.3 ± 0.5	4.1 ± 0.3	6	8
5	20.8 ± 0.3	2.1 ± 0.3	6	8

*Statistical evaluation of baseline values showed that the difference between first and second day is not significant, after that the baseline values are significantly higher with each successive application.

mmHg on the fifth day. On the fifth day the baseline was 50% higher than on the first day. This suggests a gradual accumulation of the drug at its site of action.

On the other hand, the relative increase in pO_2 after successive applications of HSL was not linear, but reached a maximum on the third day and then remained almost constant. It is possible that the vessels were already in a state of increased dilation before the next application of BN and that further dilatation after treatment with the drug was not possible.

The changes in the time when BN starts to act are presented in **Table 2**. The values of t_{lag} and t_{max} decrease on repeated application to a steady value after 3 days. The increase in the observed rate of response could be due to the induction of enzymes that hydrolyze BN to active nicotinic acid.

A similar response to repeated application was also observed using extruded HSL liposomes. The t_{lag} and t_{max} of BN action after daily repeated application in extruded and non-extruded NSL liposomes, and of free BN in hydrogel, were 5 to 10 minutes shorter than the corresponding values shown in **Table 2**. Their baseline and ΔpO_{2max} were similar to data for nonextruded HSL liposomes (**Table 2**).

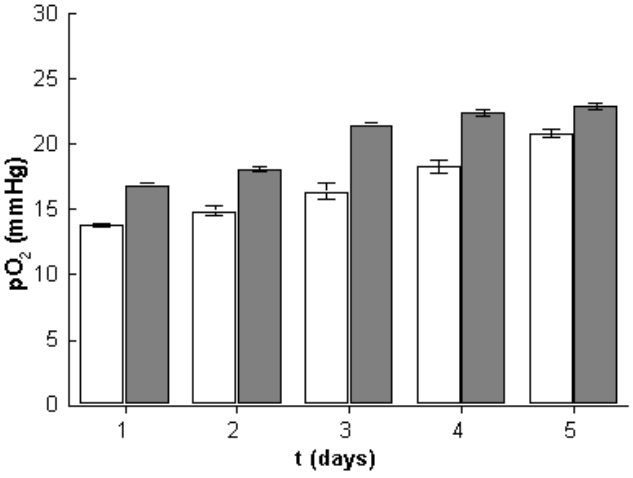

Figure 5. Influence of repeated topical application of BN in HSL liposomes over a period of 5 days on pO_2 in mouse skin: daily baseline (empty columns) and maximal pO_2 after application (full columns), (n = 3; values ± SEM).

CONCLUSION

EPR oximetry has been shown to be a suitable method for observing skin oxygenation in vivo after application of a vasodilator drug. It can be used for following the influence of different drug delivery systems on the time course and their effectiveness. We conclude that liposomes lead to faster BN penetration into the skin than a hydrogel carrier. Furthermore, the liposome composition and size play an important role in dermal penetration of a lipophilic drug. The results of repeated application show that a vasodilator, together with the appropriate carrier, can achieve a greater effect in a short time. Our results demonstrate that EPR oximetry in vivo is a very sensitive method and that small differences in pO_2 can be resolved. The possibility of detecting such small changes in pO_2

could be especially important in the radiotherapy of tumors, since the response of tumors is affected by the local concentration of oxygen. Pretreatment of a tumor with BN before radiotherapy could contribute significantly to the success of tumor therapy. The liposomal drug delivery system developed here enables controlled release of vasodilator and would allow an appropriate time for beginning irradiation treatment to be defined. This marks a new possibility for nicotinate use in therapy.

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